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Effects of cortisone and allied adrenal steroids upon limb regeneration in hypophysectomized *Triturus viridescens*

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Eight figures.

Experiments reported in a recent paper (Schotté & Chamberlain 1955) show that the administration of ACTH to hypophysectomized newts leads to the restoration of regenerative processes in amputated limbs otherwise incapable of regeneration in absence of the pituitary (Schotté 1926; Richardson 1940 and 1945; Hall & Schotté 1951).

It is fairly certain from recent findings in mammalian endocrinology that ACTH does not act directly upon the target organ, but that the secretion of this hormone under stress (Selye 1947 and 1950) stimulates the release from the adrenal gland of cortical substances which may affect directly the injured area. As was stated by Long 1950, " any study of the regulation of the adrenal cortical secretion to a large degree resolves itself into a study of the factors responsible for the release of ACTH from the anterior pituitary ". It is furthermore noteworthy that in mammals and in man traumatic stress in the form of an inflicted wound has been shown to affect the pituitary-adrenal syndrome (Upton & Coon 1951; Baker & Whitaker 1948; Ragan, Howes, Plotz, Mayer & Blunt 1949; Behrmann & Goodman 1950; Creditor, Bevans, Mundy & Ragan 1950; Baker 1950; Clayton & Prunty 1951).

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Moreover, these and other authors have shown that ACTH and cortisone are for all intents and purposes interchangeable as regulators, in an inhibitory sense, of wound healing processes.

There are no known endocrinological experiments to prove that the pituitary-adrenal synergism is operative also in amphibia, but in view of the fact that the general response patterns of amphibia toward hormonal agents are in most respects comparable to those of other vertebrates it is reasonable to make this assumption. If this be the case, then our experiments having shown that replacement therapy with ACTH causes recuperation of regenerative potencies in absence of the pituitary suggest, but do not yet prove, that cortical hormones might, under similar conditions, be equally effective.

The following experiments dealing with the administration to amputated hypophysectomized newts of total adrenal extracts, of cortisone and of desoxycorticosterone (preliminary results already reported by SCHOTTÉ 1953) represent only a first attempt to throw some light upon the weighty problem of the existence, in amphibia, of a pituitary-adrenal synergism causally related to regeneration.

MATERIALS AND METHODS

All the experiments were performed on adult newts, *Triturus viridescens*, collected from ponds of Western Massachusetts. The hypophysectomies were invariably performed previous to the injections, and the amputations of the forelimbs were effected either simultaneously with the pituitary operation or coincidentally with the first administration of the hormonal preparations. All animals were kept, for the duration of the experiments, under constant temperature conditions at 20° ± 1° C.

Three commercially available adreno-cortical preparations were used: (1) *Total adreno-cortical extract* (Eschatin, Parke, Davis and Co.). Each ml of this preparation, which was used full strength, is of a biological activity equivalent to .2 mg of 11-dehydro-17-hydroxycorticosterone and contains 0.5% phenol as a preservative; (2) *Cortisone* (11-dehydro-17-hydroxycorticosterone-21 acetate, Cortone of Merck and Co.) at a concentration of 25 mg per ml. This

was administered either full strength or diluted a hundred-fold with distilled water to 0.25 mg per ml; (3) *Desoxycorticosterone acetate* (Cortate of Schering Corporation). This drug suspended in sesame oil with a preservative at a concentration of 5 mg per ml was used full strength.

The site of injections varied by necessity in order to avoid deleterious effects. They were begun in the muscles of the back and shifted to the thigh, shoulders, neck and other places. Yale tuberculin syringes, .5 or .25 ml with 1/100 ml divisions and 27 gauge 1/2″ needles were used.

Since morphological results were often not detectable on gross observation, the work entailed much histological verification both of the limbs and of the heads. A total of 167 limbs were sectioned in addition to 26 control limbs. Moreover, 64 heads were sectioned and studied on slides to check for presence or absence of pituitary.

Physiological Effects of Adrenal Steroids Upon Hypophysectomized Newts.

The injection of the three above-mentioned cortical hormones produced far-reaching effects which brought about the premature death of many experimental animals well before they could show any results. Most of the syndromes observed were very similar to those which have been described after administration of cortisone to human patients and to other mammals (Sprague, Power & Mason 1950; Boland 1952).

The first and most noticeable effect, particularly after administration of Eschatin or Cortisone was sluggishness of the newts, beginning a short time after the first injection and attributable according to Sprague to the rapid loss of potassium with subsequent weakness and fatigue. After several injections and especially immediately before death, there is a marked edema of the neck and lower jaw of the animal, due, as in humans, to an excessive increase in sodium concentration and, therefore, of water retention (fig. 1). It is possible that death is mainly due to interference with the respiratory mechanisms of these animals, since the jaw becomes completely immobilized. Even before the immobilization of the jaw, the newts lost appetite and actually swam away from food brought near them.

That the skin glands were also affected was recognizable by excessive mucous secretions which made the animals very slippery. Correlatively with changes in the skin, marked effects upon the peripheral circulation were also observed: the blood vessels (see

FIG. 1.
Photograph of adult *Triturus* fifteen days after administration of Cortisone. Note general edema, swollen neck and jaw, paleness of skin and scarcity of blood vessels.

fig. 1), while still discernible, showed, on inspection under the dissecting microscope, absence of discrete red blood corpuscles, plasmolysis and general stasis of circulation. Examination of the adrenals after prolonged adreno-cortical therapy showed, as has often been reported in human patients, general atrophy. We have made, however, no special investigations to determine the extent of the damage done to the scattered adrenal islands. As a result of these disturbances it is not surprising that within fifteen days over 50% of the animals injected had died and that after twenty

days of treatment the mortality rate rose to 80 or even 90% of the animals in some of a series which had been started with twenty or twenty-five individuals. It was difficult to maintain a few cases alive for longer than twenty-five days.

EFFECTS OF CORTICAL HORMONES UPON REGENERATION IN FORELIMBS OF HYPOPHYSECTOMIZED NEWTS

The question posed in the introduction will be considered answered in a positive sense if replacement therapy with cortical hormones brings about regeneration in amputated limbs in hypophysectomized newts. However, in view of the pitfalls relative to pituitary operations which have recently been revealed by Hall & Schotté, *op. cit.*, in every case in which after administration of cortical hormones regeneration is observed after hypophysectomy, the completeness of pituitary removal was verified on slides by a thorough search of the cranial cavity for pituitary remnants. Such fragments were found in a few cases, but our statements, demonstrations and conclusions are based only on those cases in which the search for pituitary remnants was found to be negative.

The conditions for positive diagnosis of " regeneration " are the following: (1) Dedifferentiative processes which may affect all or only some of the stump tissues—bone, cartilage, connective tissue and muscle—must be discernible. (2) The presence of an appreciable amount of dedifferentiated, blastematous cells, or the formation of a typical blastema must be established. (3) At least in the early stages of blastema formation, there should be no dermal tissue present separating the wound epithelium from the cut old tissues of the stump.

The extent of dedifferentiative processes and hence the visible accumulation of dedifferentiated cells into a blastematous mound depend naturally upon the time which elapses between amputation and fixation. Under the conditions of these experiments, and for reasons which will become evident, however, there is no direct relationship between the amputation age of a particular limb and its degree of regeneration.

I. Effects of total adreno-cortical extracts upon regeneration in hypophysectomized newts.

After hypophysectomy with concomitant amputation of one or two limbs the first injections of Eschatin (Parke and Davis) were administered. For the first three days .1 ml was injected each time. After this an oily pool of injection mass was observable beneath the skin and the administrations were interrupted for several days. When resumed, the dosages were cut in half and the injections performed only every two or three days, the health and general appearance of each newt being the only criterion for the frequency of injections. Innumerable cases of death during the injection process were observed.

A total of 19 animals (38 limbs) survived to provide observable results and 16 limbs were studied on slides: three were fixed earlier than fifteen days with uncertain results and the other thirteen were fixed from fifteen to twenty-eight days after amputation. Among these latter cases regeneration was found to be absent on slides in four cases, while in the remaining nine cases regeneration in hypophysectomized animals became apparent as early as fifteen days after amputation.

That replacement therapy with this cortical extract cannot under these conditions be considered equivalent to the reestablishment of a normal hormonal equilibrium is exemplified by case 169 R (fig. 2). This limb was fixed 25 days after amputation and the beginning of injections; completeness of pituitary removal for this case was verified on slides by thorough search of the cranial cavity; nine injections representing a total of .7 ml of Eschatin were administered. Figure 2 shows a longitudinal section of the right limb and the presence of a blastema according to the above enumerated criteria is unmistakeable. If this regenerate is compared with figure 3, representing a normally regenerating control animal of the same amputation age, it is evident that the restitution of regeneration in the limb of the treated animal is partial and substantially retarded. The possible causes of retardation and of total absence of regeneration in some limbs will be discussed after consideration of the other experimental cases. However, no matter how retarded or how imperfect the regeneration, it is

FIG. 2.

Photomicrograph of right limb of case 169 R, fixed 25 days after hypophysectomy, amputation and Eschatin injections. Note absence of dermal formations and of skin glands in center of amputation area, also presence of blastematous cells. The somewhat shredded aspect of epithelium indicates that effects of hypophysectomy were not completely overcome. Magnif. × 40.

FIG. 3.

Typical regenerate of control limb after 25 days of regeneration. Note absence of dermis in blastema region and differentiation of procartilaginous skeletal formations. × 40.

important to emphasize, at this place, that in nine cases a recuperation of regenerative capacities, in ascertained absence of the pituitary, has been obtained. These results, following relatively haphazard injections of a total extract of adreno-cortical hormones have led to the study of the effects upon regeneration of two other cortical steroids.

2. Effects of cortisone upon regeneration of the forelimb in hypophysectomized newts.

The general procedure of hypophysectomies, amputations and injections was the same as in the preceding series. In order to detect possible effects of dosages six different quantities or dilutions were used. In utilizing Merck's normal concentration of Cortone, .1 ml, .05 ml, .02 ml or .01 ml were injected to four different groups. In addition, a diluted concentration of commercial Cortone was administered also in dosages of .02 or .01 ml. Because of the severe ravages which were observed, daily injections were interrupted for one or two days and subsequent administrations were resumed whenever the subcutaneous injection mass was judged to be sufficiently depleted. After twenty days, the animals were in such a bad shape that additional injections could be administered only every fifth or sixth day. A total of eighty-eight animals received cortisone injections, from which group 89 limbs, in addition to the control limbs, were studied histologically. A computation of all the cases arranged according to the strengths and duration of cortisone treatment is presented on Table 1.

The data indicate that while there is no discoverable relationship between the amounts of cortisone received and amounts of regeneration, they also show that regeneration has been declared absent in all the cases (48 limbs) which were fixed earlier than twenty days after amputation, a statement which demands qualification. A normal newt, kept at 20° C, presents fifteen days after amputation a well-developed blastema, but after hypophysectomy and simultaneous amputation complete cicatrization with dermal invasion always occurs at this time (Consult figure 3, Hall & Schotté, *op. cit.*). In cortisone injected hypophysectomized newts, however, fixed fifteen days after amputation our histological studies show that the epidermis covering the amputation surface

TABLE 1.

Histologically Confirmed Effects of Various Concentrations of Cortisone Upon Regeneration of Fore Limbs in Hypophysectomized Newts

Days of Regeneration with Cortisone	No. of Limbs	Amounts Administered at Normal Concentration								Amounts Administered After Dilution				Totals	
		.1 cc.		.05 cc.		.02 cc.		.01 cc.		.02 cc.		.01 cc.			
		Diagnosis of Regeneration on Slides								Status of Regeneration				Reg. Pres.	Reg. Abs.
		Pres.	Abs.	Pres.	Abs.	Pres.	Abs.	Pres.	Abs.	Pres.	Abs.	Pres.	Abs.		
3 to 10 days	48	0	12	0	22	0	4	0	4	0	3	0	3	0	48
20 to 28 ..	26	3	1	2	3	3	1	4	4	0	2	0	3	12	14
29 to 46 ..	15	7	0	3	0	1	0	3	0	1	0	0	0	15	0

is still devoid of dermal lining and the cartilaginous tip of the radius (and also of the ulna) shows evidence of dedifferentiation. There are, in addition, giant cells and perhaps some scattered blastematous cells, probably originating from mesenchymatous elements. Among the 48 limbs of this amputation age studied, twenty present this aspect which, while not being one of a freely regenerating limb

FIG. 4.

Photomicrograph of case CB 63, in absence of pituitary regenerating after thirty-three days of cortisone treatment. Note perfect blastema comparable to control case in fig. 3. × 40.

nevertheless is not one of regeneration permanently blocked. The " freezing ", so to speak, of regenerative processes after replacement therapy has been observed and described in similar experiments dealing with the administration of ACTH to amputated normal and hypophysectomized newts (see particularly figs. 3 and 4, SCHOTTÉ & CHAMBERLAIN 1955).

In contrast to the above cases, all fixed earlier than twenty days after hypophysectomy, amputation and cortisone administration there is ample evidence that regenerative processes are reawakened under those conditions if the newts survive for longer periods. Our evidence shows that in animals fixed during the third week

after amputation obvious regeneration has occurred in about fifty percent of the cases and, finally, regeneration according to the above enumerated criteria is diagnosed for all cases which have survived the injections for more than four weeks.

Excellent regeneration in histologically proven absence of the pituitary is shown by one such case CB 63, fixed 33 days after amputation and cortisone treatment (fig. 4). The advance in regeneration of this limb over the one previously illustrated (fig. 2) is obvious: the typical palette stage of the regenerate indicating the beginning of external morphogenesis was macroscopically observed and the early differentiation of procartilaginous prongs can also be followed on slides. The retardation of this and of similar cases in respect to control limbs of the same amputation age which was so conspicuous in the treated animals fixed earlier is, at this later age, reduced to not more than a matter of five days. It appears, then, that regeneration once started proceeds at an approximately normal pace.

Macroscopical observations on eighty-eight animals and the study on slides of eighty-nine limbs from animals for which the absence of pituitary has been verified in every case show, therefore, that the injection of cortisone tends to nullify the effects of hypophysectomies:

(a) In all cases in which the limbs were fixed earlier than twenty days after amputation, the processes of early blastema formation are merely retarded; even when no regeneration occurs the typical histological features of non-regeneration observed in previous investigations of hypophysectomized newts with concomitant amputation fail to materialize—there is no dermal invasion and no formation of a hardened basal membrane, thus suggesting that regenerative processes are merely suspended, not blocked.

(b) The twenty-seven cases which after injection of cortisone exhibited unmistakable regeneration of their limbs in ascertained absence of the pituitary also showed that the replacement therapy with cortisone is contingent with a considerable delay in the initiation of regenerative processes. This delay becomes less noticeable for the animals which lived a month and longer.

(c) The substitution of cortisone for the totality of pituitary hormones is not always effective, as is proved by absence of regeneration in fourteen among forty-one cases in which regeneration might have become possible.

(d) There is no evidence that the recuperation of regeneration in limbs of hypophysectomized and cortisone treated animals leads to defective regeneration, as is proved by normal histogenesis and structurally normal blastemata.

3. Effects of the desoxycorticosterone fraction of the adrenal cortex upon regeneration in limbs of hypophysectomized newts.

Commercial Cortate (Schering Corporation) was used undiluted in dosages of .15 ml, 1.0 ml and .05 ml. These dosages were administered in a manner similar to that which has been described for the cortisone series. Presence of the injection substance was ascertained by the oily swelling beneath the skin and injections of additional dosages, while appreciable amounts of substance were still detectable led invariably to speedy death of the animal. Forty-five animals were studied and fifty-two limbs were examined on slides. No differences in regeneration were detectable in respect to the three dosages administered.

The results are comparable to those presented in the previous series: thirty-three limbs which were fixed after a regeneration age of 4 to 17 days showed no regeneration; but among the 19 hypophysectomized newts which were fixed from 18 to 32 days after the Cortate injections and amputations of one or two limbs, ten presented regeneration, all of them with considerable retardation in respect to controls.

As an example of recuperation of regeneration with desoxycorticosterone injections, a section of the limb of case CB 54 (fixed 32 days after amputation and hypophysectomy, and having received in five injections a total of .25 ml of Cortate) is illustrated in fig. 5. The limb is definitely regenerating: the aspect of the epidermis, the presence of blastematous cells, dedifferentiation of muscle fibers and of the bone shaft are all signs of a belated (by about two weeks) recuperation of regenerative processes. None

of the other cases presented any regeneration which was further advanced. Limbs from this series which two weeks after amputation exhibited no blastema formation have much in common with the already described state of retarded preregeneration: as in those cases there are no blastematous accumulations, but there are no visible dermal pads either, and the amputation tips present an

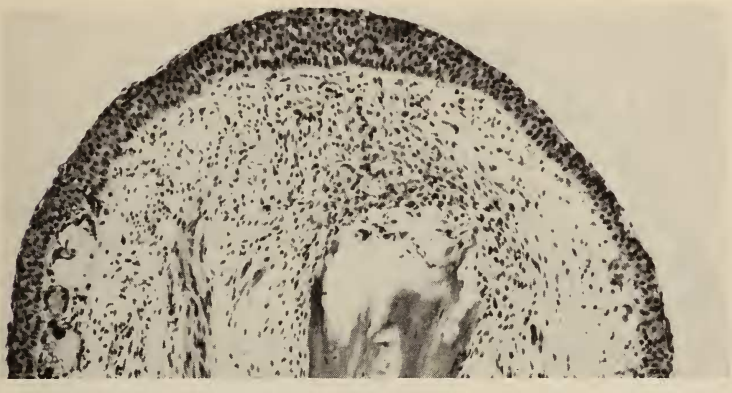

FIG. 5.

Photomicrograph of case CB 54, thirty-two days after hypophysectomy, amputation and desoxycorticosterone therapy. Note absence of dermal lining of wound epithelium, unmistakable dedifferentiation with formation of blastematous cells and penetration at left of the bone shaft of a large nerve traceable to the very tip of the accumulation blastema. Comparison with twenty-five days normal regeneration in figure 3 shows retardation of processes. × 40.

aspect of temporary arrest rather than one of final inhibition of regeneration.

Tentative interpretation of retarded and of inhibited regeneration after replacement therapy with cortical hormones.

The conspicuous retardation in respect to controls was not understood until newts with intact pituitaries showed similar retardation in the initiation of regenerative processes after cortisone injections. Normalcy in regeneration, it becomes more and more evident from these and other studies, is the result of a delicate balance of a host of conditions within the regenerating animal. Disturbances within this equilibrium, be it by excess or by want

of one or several hormones, or by stress conditions (traumatic, chemical or emotional) are all factors which have been shown to interfere with the normal course of regeneration. It was mentioned before that administration of massive doses of cortical extracts, about the pharmacology of which in amphibia we know next to nothing, determines a general upset in the health of newts leading

FIG. 6.

Photomicrograph left limb of case CC 30, fixed twelve days after hypophysectomy and amputation and after repeated cortisone injections. Note precocious invasion of dermal formations including skin glands with complete blockage of dedifferentiation. × 40.

within a few weeks after hypophysectomy and therapy to death of the majority of the treated individuals. It is not surprising, then, that such generalized disturbances within the organism should also lead to imbalances detrimental to the wound healing processes initiating regeneration.

Retardation of regeneration in treated animals is intimately related to the problem of *non-regeneration after administration of cortical hormones.* These fall naturally into two categories—regeneration temporarily suspended and regeneration blocked. Regeneration suspended is merely a retardation since our experiments show that, after cortisone treatment, all animals which have survived hypophysectomy and cortisone administration for over twenty-five days show regeneration (see section 2). An entirely

different condition exists in other cases in which complete arrest of regeneration can be shown on slides. One such case is represented by animal CC 30 and the section of its limb is illustrated in figure 6. The photomicrograph of this limb fixed twelve days after amputation, hypophysectomy and cortisone administration clearly shows that a thin pad of dermal tissue with glands is lining the full extent

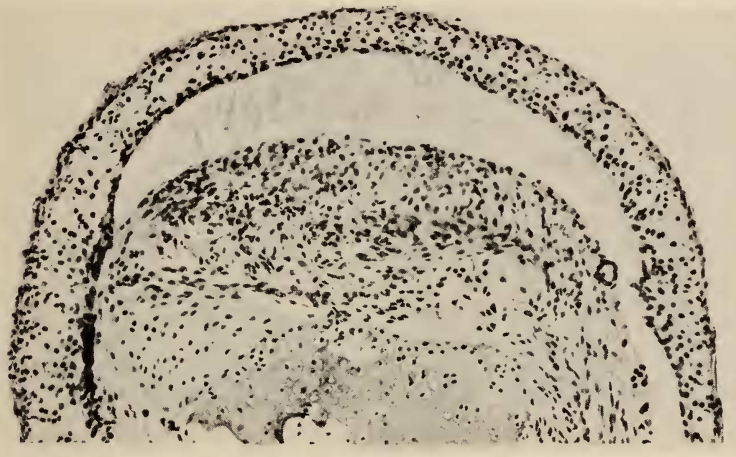

Fig. 7.

Photomicrograph of case Esch 9 after twenty-nine days treatment with cortical extracts. Note formation of subepithelial space with complete separation of epidermis and a separate mass of dedifferentiating cells lying across amputation stump. × 40.

of the amputation stump, thus effectively separating the wound epithelium from the raw tissues of the amputation surface. There is moreover, a thin lymphatic space which, in the case of anura (see Schotté & Harland, 1943, figs. 12, 13 and 16; and also Forsyth, 1946, fig. 17) is concomitant with non-regeneration in older tadpole limbs. The existence of a dermal pad likens this case to the aspect which has been observed and described for all cases in which regeneration becomes impossible after hypophysectomy performed simultaneously with amputation (see Hall & Schotté, 1951, fig. 3, 7 and 8). Why a dermal pad should be formed so precociously must remain unexplained.

Additional disturbances directly attributable to the effects of injections, particularly with total adreno-cortical extracts (Escha-

tin), are evidenced by blisters and subepidermal or subcutaneous spaces which were observed macroscopically and on slides in a dozen limbs. Such a condition is represented by the case Esch 9 kept under observation for twenty-seven days. The figure 7 shows that the epidermis with its stratum germinativum is completely separated from the connective tissue by a vast lymphatic or other liquid phase. There can obviously be none of the indis-

FIG. 8.

Photomicrogaph of case CB 80, a cortisone case, hypophysectomized and amputated twenty-eight days previous to fixation. Note shredded aspect of epithelium and presence of considerable amounts of dedifferentiated cells intermingled with debris and connective tissue masses. × 40.

pensable " epidermis-cut tissue " relationship so important for the normal course of blastema formation. The cells situated distally from the cut bones exhibit dedifferentiative activities, but there is no possibility for these cells to form a regular blastema because other conditions for blastema formation are wanting.

Finally, it will be recalled that in these experiments most of the newts previously deprived of their pituitaries were injected with cortical steroids in a somewhat irregular fashion, and since these steroids, as we propose to show in another paper, interfere even with normal regeneration, it is not difficult to understand that disturbances within the delicately balanced phases of regeneration may occur. An interesting example of interference with the regular phases of early regeneration is given by case CB 80 of an hypophysectomized newt which had its forelimb amputated

twenty-eight days previous to fixation (a total of 0.225 ml of Cortisone was administered). The photomicrograph (fig. 8) clearly shows that, while there is abundant evidence of dedifferentiation, blastematous cells are dispersed within the maze of adult connective tissue cells, thereby disturbing blastema formation. It is clear to the experienced student of regeneration that at one particular time the unstable equilibrium of " Cellular interactions " (Butler & Puckett, 1940) has become detrimental to normalcy in regeneration. It can surely not be expected that a haphazard injection of cortical hormones should not only reestablish " normal " hormonal equilibrium, but that it should also restore the requisite timing in these hormonal interactions. If one recalls that in many cases, in order to keep the animals alive, the injection procedures had to be interrupted and the dosages lowered, then the occurrence of disturbances in the restitution of regenerative processes in some limbs of hypophysectomized newts becomes plausible. The " shredded " aspect of the epithelium on figure 8 points to just one such possible interpretation, since it proves that the replacement therapy by cortical hormones was not effective in changing the aspect of the skin of a hypophysectomized newt from the rugged and shredded to the smooth and slippery appearance described for the newt represented on figure 1. It is, therefore, not surprising to observe that regeneration did not take place in this and in similar cases.

DISCUSSION

The above experiments have shown that the administration of cortical extracts to amputated hypophysectomized newts may be followed either by full recuperation of regenerative powers or by regeneration after considerable delay; they have also shown, however, that in many cases the replacement therapy used was incapable of modifying the status of non-regeneration expected after hypophysectomy. The wide divergence of results points to the inevitable conclusion that the initiation of regenerative processes is an eminently labile process rather than a yes or no proposition.

This investigation was undertaken after detailed histological examinations had revealed (Hall & Schotté, *op. cit.*) that non-regeneration in newts after hypophysectomy was attributable to

precocious dermal invasion blocking the initial phases of regeneration. Yet, wound healing or its retardation is under cortical hormonal control, since it has been shown for mammals at least, that the administration of cortisone or of ACTH interferes with this process in delaying the formation of granulation tissue. The proposition that cortical hormones are involved in regeneration rests, therefore, on the assumption that in the newt, as in mammals, the stress of amputation sets into motion the general adaptation syndrome with concomitant release of cortical hormones. If the GAS syndrome is extant in amphibia, then it becomes understandable why replacement therapy with cortical hormones should restitute regenerative powers in amputated newts in spite of the absence of the pituitary.

No experimental proof of the existence of a pituitary-adrenal synergism in amphibia has been given. Yet, the results from these experiments confirm the assumption made in the introductory part of this paper, since the histological evidence indicates that premature closure of the amputation surface is indeed prevented by the administration of cortical contracts.

There are further supports for the hypothesis of cortical involvement in the regeneration of newts:

(1) Recent experiments reported by SCHOTTÉ & CHAMBERLAIN, *op. cit.*, proving that ACTH, administered to hypophysectomized newts restores the regenerative capacities of these animals, are surely supporting evidence of the existence of a pituitary-adrenal synergism efficient in regeneration. It is difficult to interpret this effect of ACTH otherwise than by its action upon the adrenal cortex. But in view of our ignorance of the pathways, particularly in amphibia, of hormonal interactions, one must remain hesitant in assuming as self-evident that the action of ACTH in regeneration can be explained only by its stimulatory role upon the adrenals.

(2) Another confirmation of the " utilization " of cortical hormones in regeneration is suggested from recent investigations by SCHOTTÉ & LINDBERG 1954 when recuperation of regeneration in hypophysectomized newts was observed following transplantation of frog adrenals. But here again caution is indicated, inasmuch as we had not offered direct proof that

frog adrenals transplanted into newts had remained functional for the few days required to bring about regeneration.

(3) Direct proof of cortisone action on wound healing in amputated limbs of normal newts was recently offered by Manner 1955, who described the inhibitory action of cortisone acetate on the mitotic activity of both epidermal and mesenchymal cells.

(4) Additional still unpublished results obtained recently at this Laboratory further support the hypothesis that cortisone may substitute for the missing pituitary in promoting regeneration. It will be shown in a forthcoming publication that hypophysectomized and amputated newts kept for over twenty days in a weak cortisone acetate solution regenerate in all cases without exception.

(5) And finally, the effects of various stressors upon regeneration provide complementary, although still indirect, proofs of the existence of a GAS syndrome in regeneration: a series of experiments from the Laboratory, published so far only in preliminary form, have shown that repeated traumatic or chemical stressors inhibit substantially and even obliterate normal regeneration in newts with intact pituitaries. In hypophysectomized newts, on the contrary, the same stressors determine recuperation of regeneration (Lindberg & Schotté 1955, Pellman & Schotté 1955 and Schotté & Bonneville 1955).

The results from all these experiments are explained tentatively by the hypothesis that in a newt with intact pituitary repeated traumatic or chemical stress elicits an excess of cortical secretions inhibiting normal regeneration, exactly as is the case after administration of ACTH or of cortical preparations from our present experiments. In contrast to this, in animals incapable of regeneration for want of a pituitary, stress of various causation, it is argued, stimulates cortical secretions in amounts which are prerequisite for proper wound healing conditions, hence regeneration is re-initiated. But here again the proper equilibrium is essential and too much cortisone, even in hypophysectomized animals, produces inhibitory effects as shown by delay and even suppression of regeneration in many cases. The mechanism by which stress alone may produce these astonishing results is so far unknown.

There is no interpretation concerning the mechanism of action of cortical hormones upon cellular activities which take place within the narrow confines of the amputation surface and upon which regeneration or its absence depends. Even for mammals there is scarcity of information on the action of cortical hormones upon cellular activities (see CAMERON's 1953 comments on the absence of basic research in that field). The thoughtful statement of LONG 1950 that "the question as to whether there is actual 'utilization' of the cortical hormone by tissues is still unproven" has not yet been contradicted. It is possible that cortical hormones are instrumental in promoting or in retarding cellular migration, perhaps even mitosis. But for want of exact information on the subject and until results from other investigations become known, we must be satisfied with a more modest conclusion:

(a) In spite of exceptional cases replacement therapy with commercially available cortical extracts is effective in proven absence of the totality of the pituitary to reestablish the cellular equilibrium requisite to set into motion the regenerative processes otherwise blocked in hypophysectomized newts;

(b) It is therefore fairly certain that a pituitary-adrenal synergism of some sort is operative in amphibian regeneration.

SUMMARY

(1) The action of three commercial adreno-cortical preparations (Eschatin- a total extract, Cortisone and Desoxycorticosterone) on the regeneration of forelimbs in hypophysectomized adult *Triturus viridescens* was studied on over two hundred animals. Histological data from 158 limbs were obtained, and in all cases in which positive results were observed (namely, regeneration in hypophysectomized newts) the corresponding heads of newts were sectioned in order to search for possible pituitary remnants (64 heads sectioned). All animals were kept at 20° C to insure comparable data on regeneration and on delays in the process.

(2) The severely deleterious physiological effects of injected cortical steroids upon newts are described. While the dosages used exceeded manifoldly the amounts employed in human therapy,

it was found that differences in concentrations of the various extracts have produced no appreciable differences in the responses of hypophysectomized newts in respect to regeneration of their limbs.

(3) Data from these experiments based almost exclusively on histological observations show that all three cortical preparations may cause limbs of hypophysectomized newts, otherwise incapable of regeneration, to regenerate again. There was, however, in every case, a delay in the initiation of regenerative processes, and there were also numerous cases in which recuperation of regenerative processes in hypophysectomized newts failed to take place. It is argued that these exceptional cases are attributable to irregularities in the hormonal balance of hypophysectomized newts caused by the poisonous substances injected at times critical for the initiation of regeneration.

(4) Since both adreno-cortical extracts and (from previous experiments) ACTH have been shown competent to elicit recuperation of regenerative properties in amputated hypophysectomized newts and since regeneration seems to depend upon a proper regulation of wound healing processes, it is tentatively concluded that a pituitary-adrenal synergism is operative as an efficient mechanism in amphibian regeneration.

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